THE EFFECT OF BODY CELLS AND FLUIDS ON CERTAIN DYES

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It is a well-known fact that certain dyes are highly toxic to microorganisms. This action is more or less specific in character so that although dianil blue is active against trypanosomes, it is almost without effect on B. typhosus. Gentian violet is germicidal to gram-positive bacteria. Also it must be borne in mind that a dye which is efficient in vitro is not of necessity of equal value in vivo. Chamberland,[1] working with oils, observed this difference in bactericidal action within and without the animal body and has been corroborated by numerous investigators. Test tube activity, however, is the best preliminary criterion thus far discovered for possible effectiveness in the body.

The usefulness of dyes for chemotherapeutic purposes is dependent on a number of processes that are of interest. These may be subdivided into (a) their germicidal value in vivo, (b) their effects on living tissues, (c) the processes by which they are removed from the circulation after introduction to it, and (d) the channels by which excretion takes place. These subjects should be considered before final judgment of the worth of a substance for purposes of treatment is rendered.

An endeavor has been made to learn whether any one of the available coloring matters is efficient for chemotherapeutic use in treatment of experimental infection by B. typhosus in rabbits. In searching for information in this field, experiments both in the test tube and in the animal, were carried out.

The particular scope of the work here outlined, which is one subdivision of that noted in the second paragraph, includes observations concerning the processes by which dyes are removed from the circulation after intravenous injection. The methods are those of the test tube rather than those pertaining to experiments made on the living animal since our knowledge of physiologic technic is not sufficiently advanced to allow the latter and more exact procedure to be used.

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[1] Ann. d. l'Inst. Past., 1887, 1, p. 152.

It was noted that while one dye may be eliminated in part through the bile, another may be found only in the urine. This of itself denotes a selective affinity in the first coloring matter for special cell structures which does not exist in the second dye. Again, one compound, as for example pyronine G, shows no visible signs of its presence in the body a few minutes after injection while proflavine stains the intercostal muscles for weeks. Fat soluble dyes like sudan III concentrate in the various storehouses of that tissue while new fast green 3 B tends to color connective tissue and parenchymatous structures. These examples suffice to indicate that decolorization of the dye in the serum follows its introduction and also that it appears to be possible that special cell structures are instrumental in separation of the coloring substance. Likewise the tissues or fluid most active may vary with the anilin derivative used.

Although coloring matters have found their principal medical use in bacteriologic technic in staining, for selective mediums and as trypanocides, Neisser and Wechsburg [2] availed themselves of methylene blue for the purpose of differentiating living from dead cells. Certain investigators have endeavored to indicate and to measure cell respiratory phenomena by the reactions of dyes. Evans and his collaborators,[3] especially Schuleman,[4] have studied vital staining and have shown that a stain in the colloidal state is toxic although when in solution it may be harmless. Likewise their more complete experiments prove that a colloidal stain is much less diffusible than one in which the particles are smaller. Evans has demonstrated that when trypan blue is administered to the circulation in tremendous doses, it is removed by a series of cell structures known as the macrophages.

Reference to Fay,[5] Wahl and Atack,[6] Bucherer or other standard texts on the chemistry of dyestuffs shows that coloring matters are built up from the leuko-base which is without tint and that intermediate to these there is another elementary material known as the color base. The distinction, at least as regards the triphenylmethane group, is one of progressive addition of oxygen to the molecule and the first two are colorless. Reduction of the dye in this rosanilin class of

[2] Ztschr. f. Hyg. u. Infektionskr., 1901, 36, p. 299.
[3] Am. J. Phys., 1915, 37, p. 243.
[4] Science, 1914, 39, p. 443.
[5] Chemistry of Coal Tar Dyes, 1911.
[6] Manufacture of Organic Dyestuffs, 1914.
[7] Lehrbuch d. Farbenchemie, 1914.

compounds results in loss of color which, if the chemical changes have not proceeded too far, may in turn be restored by the introduction of hydrogen peroxide or bubbling oxygen.

The surmise stated in the fifth paragraph was followed and an endeavor was made to study some changes induced when suspensions of living cells and dilute dyes are brought together. It will be understood that the resulting effect on the dye itself rather than that on the cell was the goal. Little attention, therefore, was paid to vital staining phenomena.

This procedure was used: Cell suspensions, defibrinated blood, blood treated with sodium oxalate and two fluids, beef bile and serum were tested. The bile was obtained from a local slaughter house. The serum was from the horse. Because of the large amount of bacterial contamination in the bile when received at the laboratories, it was autoclaved at 15 pounds' pressure for 20 minutes. The serum was unaltered since it had been drawn aseptically at the Citter Laboratories in Berkeley and was furnished through the courtesy of that company. All other fluids and cell structures were from the rabbit.

Rabbit cell suspensions were made from organs removed immediately after the death of the animals, which in all cases had been killed by exsanguination. While the removal was not made under aseptic conditions, no gross contamination occurred. In some instances it was necessary to postpone use of materials until the following morning but in the meantime they were stored on ice in sterile dishes. The cells therefore were probably living at the time the suspension was made. Organs obtained were macerated in mortar with pestle in sterile physiologic salt solution. The mortar was used rather than a grinding machine because it was desired to procure an ultimate suspension as free from other cells, such as connective tissue, as possible. Red blood corpuscles (R B C) were prepared by centrifuging the defibrinated, sterile blood and then washing the sediment three times by whirling in sterile salt solution. The necessary mass was then added to fresh salt solution by means of a sterile pipet. The muscles utilized were the heavy ones from the region of the thigh.

The concentration of cells in the sodium chlorid solution was such that when settling had taken place after a few moments, the layer on the bottom of the tube in thickness was $\frac{1}{6}$ to $\frac{1}{7}$ of the total height of the column of liquid. Large particles were excluded by pouring the mixture into a conical glass and allowing them to settle out for a few seconds. Agglutination tubes were used in this series of experi-

ments and into each was poured from 1 to 1.5 c c of the suspended cell material. Sufficient dye solution was then added to each to give a decided tint after which they were shaken gently to give even mixing throughout the contents. The amount of dye in the tubes varied according to its color intensity but the limits were between 1 : 15,000 and 1 : 30,000. Finally, paraffin oil was overlaid in the tubes to exclude free circulation of oxygen after which incubation at 37 C. followed for 24 hours. When bile and serum were studied, the color intensity of the dye used was the same as that above noted.

At the end of the specified incubation period, observations were made using for purposes of comparison, when it seemed advisable, a check tube to which no dye had been added and which had received the same treatment in other respects as the remainder of the series. In some instances, sufficient material had collected against the side of the tube so that accurate observation of the remaining supernatant fluid could not be made and in such cases gentle centrifuging was resorted to in order to clarify it.

In making notes of the final condition of such preparations, it was evident that there were varying degrees of color intensity remaining in the supernatant liquid and that many preparations yielded cell sediments which were heavily colored while others retained their original and natural shade. Such color as they had taken up from the dyed suspension had been lost. Certain tubes resulted in entire loss of color both in the supernatant and in the cell layer at the bottom showing that not only had all the dye added been removed, but also that it had been destroyed. In a few instances, as with certain tubes containing methylene blue, the subsequent addition of hydrogen peroxide caused a return of the color with its former intensity, thus proving that in these samples the dye had been broken down to the leuko-base. In instances in which the color was not brought back by this reagent, it seems fair to assume that the destruction had progressed to some point below that of the leuko-base. In addition, microscopic examination was made of the detritus remaining at the bottom of the tubes and in a majority of instances no staining of the cells themselves could be demonstrated. On the other hand, clouds of stain could be seen dimly in masses of cell structures although no internal absorption of the dyestuff was evident either in nucleus or in cytoplasm. This may indicate that in these samples the stain had been taken up on the surfaces of the cells and later destroyed at that site by physical or chemical means of by both. It is quite possible that this action

was effected through the agency of cell substance by adsorption which probably means the formation of ephemeral and unstable combinations at the surface.

The results of this series of experiments with dyes in contact with living cells in vitro is given in the table. "Supernatant" refers to results of observations made on the overlying fluid. The following abbreviations and characters are used for ease in tabulation: "R B C" means red blood corpuscles; "Des." (destroyed) indicates no color remaining; "Tr." means trace; "N C" (no change) shows that no appreciable color intensity was lost in the supernatant. "Fair" is adopted as meaning some degree between no change in color and a trace. The term " S D.," used in connection with brilliant green and bile, refers to slight darkening which was the outcome in that special instance. "Yel." means yellow.

THE EFFECTS OF CERTAIN CELLS AND FLUIDS ON DYES AS SHOWN BY CHANGES IN THE SUPERNATANT FLUID

Stains	Bile	Serum	Red Blood Cells	Defib-rinated Blood	Oxa-late Blood	Brain	Mus-cle	Lung	Mar-row	Liver	Spleen	Kid-ney
Acriflavine	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
Basic fuchsin	N.C.	Fair	N.C.	N.C.	N.C.	N.C.	Tr.	N.C.	Fair	Fair	N.C.	Des.
Benzoazurin	N.C.	N.C.	.....	N.C.	N.C.	.....	.....	Tr.	Des.	Des.	N.C.	Fair
Brilliant green	S.D.	N.C.	N.C.*	Tr.	Tr.	N.C.	Des.	N.C.	Des.	Tr.	Tr.	Tr.
Congo red	N.C.	N.C.	N.C.	N.C.	N.C.	.....	.....	Fair	Tr.	Des.	Fair	Fair
Corallin	N.C.	Fair	Tr.	Tr.	N.C.	.....	.....	Des.	Des.	Des.	Des.	Des.
Crystal violet	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	Tr.	N.C.	Tr.	Des.	Des.	Tr.
Cyanin B	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	Tr.	N.C.	N.C.	Tr.	Des.	N.C.
Erioglaucin A	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	Tr.	N.C.	Fair	Tr.	N.C.	Tr.
Malachite green	N.C.	Tr.	N.C.*	N.C.	Br'wn	N.C.	Des.	N.C.	Tr.	Des.	Des.	Tr.
Methylene blue	N.C.	Fair	N.C.	N.C.	N.C.	N.C.	Des.	Fair	Fair	Des.	Des.	Des.
Methyl violet 5B	N.C.	N.C.	Tr.	N.C.	N.C.	.....	.....	N.C.	Tr.	Des.	Fair	Fair
Neutral red	N.C.	Tr.	.....	N.C.	Fair	.....	.....	N.C.	Yel.	Yel.	Tr.	Yel.
New fast green, 3B	N.C.	Fair	N.C.	N.C.	N.C.	N.C.	Tr.	N.C.	Tr.	Des.	Fair	Tr.
Oxamine violet	N.C.	N.C.	N.C.	N.C.	N.C.	.....	.....	Fair	N.C.	Des.	Tr.	Tr.
Proflavine	N.C.	N.C.	.....	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.
Pyronine G	Fair	Tr.	.....	N.C.	N.C.	.....	.....	Tr.	Tr.	Tr.	Des.	Des.
Safranin	N.C.	N.C.	N.C.	N.C.	N.C.	.....	.....	Fair	Tr.	Fair	Fair	Tr.
Saüregrün	N.C.	N.C.	N.C.*	N.C.	N.C.	N.C.	Des.	Tr.	Tr.	Des.	Fair	Des.
Setocyanin	N.C.	N.C.	Tr.	N.C.	N.C.	N.C.	Des.	Fair	Des.	Tr.	Des.	Tr.
Spiller's purple	N.C.	N.C.	N.C.	N.C.	N.C.	N.C.	.....	Fair	Tr.	Fair	Fair	Fair
Trypan blue	N.C.	Fair	N.C.	N.C.	N.C.	N.C.	Des.	N.C.	Des.	Des.	Tr.	Des.

* Cells disintegrated.

It should be considered that it is not impossible that the changes noted may be connected with cellular respirational function of interchange of gases. The mechanics of removal, however, have not been explained.

SUMMARY

On reading the table it will become evident that when brought into contact with serum, bile and brain, little change was wrought in any

of the coloring matters. On the other hand, certain secreting and filtering tissues, such as liver, kidney, bone marrow and spleen, showed a high degree of activity. Lung and muscle occupy an intermediate position. Certain dyes such as acriflavine and proflavine showed no change throughout. Other stains varied in outcome according to the particular cell structure with which they were placed in contact. Some dyes were reduced to the leuko-base as methylene blue many times and as trypan blue once. Others were changed into products further removed from the original molecular structure. It seems sufficiently conservative to offer the following deductions:

Dyes are removed from the circulation through the agency of cell structures. Tissue cells rather than blood corpuscles are instrumental in this respect since neither serum, washed corpuscles, defibrinated blood nor blood treated with sodium oxalate is active as a decolorizing agent in vitro.

The particular tissue instrumental in this removal varies with the dye in question.

Separation of the dye from its solution in the serum is brought about by adsorption on cell surfaces with no vital staining evident at concentrations used.

Stains thus removed by adsorption are destroyed.

This molecular destruction of the dye continues to the production of the leuko-base in a few instances only, while in a great majority it extends to the formation of products further removed from the original chemical structure.